

EVIDENCE OF APOPTOSIS IN ARRHYTHMOGENIC RIGHT VENTRICULAR DYSPLASIA

ZIAD MALLAT, M.D., ALAIN TEDGUI, Ph.D., FABRICE FONTALIRAN, M.D., ROBERT FRANK, M.D., MICHEL DURIGON, M.D., AND GUY FONTAINE, M.D., Ph.D.

ABSTRACT

Background Arrhythmogenic right ventricular dysplasia, a disorder that may lead to severe ventricular arrhythmias and sudden death, is characterized by the progressive replacement of myocardial cells by fat and fibrous tissue. We examined whether the loss of myocardial cells in this disease could result from cell death by apoptosis (programmed cell death).

Methods Specimens obtained at autopsy from the right ventricular myocardium of eight patients with arrhythmogenic right ventricular dysplasia and four age-matched normal subjects were analyzed. To identify individual cells undergoing apoptosis, we performed in situ end-labeling of fragmented DNA on paraffin sections using biotinylated deoxyuridine triphosphate and the enzyme terminal deoxynucleotidyl transferase. We also examined the level of expression of CPP-32, a cysteine protease required for apoptotic cell death in mammalian cells, using immunohistochemical techniques.

Results Apoptosis was detected in the right ventricular myocardium of six of the eight patients with arrhythmogenic right ventricular dysplasia and was absent in the controls. High levels of expression of CPP-32 were associated with positive in situ endlabeling of fragmented DNA.

Conclusions These results indicate that apoptotic myocardial cell death occurs in arrhythmogenic right ventricular dysplasia and may contribute to the loss of myocardial cells in this disorder. (N Engl J Med 1996;335:1190-6.)

©1996, Massachusetts Medical Society.

POPTOSIS, or programmed cell death, is a highly regulated and active process that contributes to the control of cell number during development and to the maintenance of many adult tissues. ¹⁻³ It is triggered by the activation of an internally encoded suicide program as a result of either extrinsic or intrinsic signals. ⁴ Apoptosis differs morphologically from necrosis: it is characterized by blebbing of the cell membrane, a reduction in cell volume, condensation of nuclear chromatin, and endonucleolytic degradation of DNA at nucleosomal intervals. ² Apoptotic bodies are digested or phagocytosed by adjacent cells or macrophages without inducing an inflammatory response.

The crucial role of apoptosis in pathologic conditions is increasingly being recognized.^{5,6} Recently, apoptosis was reported as a possible mechanism for

the loss of myocardial cells in an infant with Uhl's anomaly.⁷ Arrhythmogenic right ventricular dysplasia, a form of right ventricular cardiomyopathy that commonly leads to severe ventricular arrhythmias and sudden death,^{8,9} is characterized by noninflammatory loss of myocardial cells and their progressive replacement by fat and fibrous tissue.⁸⁻¹¹ We hypothesized that this loss of myocardial cells in arrhythmogenic right ventricular dysplasia may result from cell death by apoptosis.⁶

METHODS

Cardiac Specimens

Sections from the right ventricle of eight patients (five men and three women; mean [±SD] age, 47±15 years) who died of arrhythmogenic right ventricular dysplasia were examined. Five of the patients had documented ventricular arrhythmias. The final diagnosis of right ventricular dysplasia was based on the following established criteria9-12: massive infiltration of the right ventricular wall by fat tissue, with surviving strands of cardiomyocytes embedded in or bordered by fibrous tissue (Fig. 1), a finding typically distinct from the patchy replacement of myocardium by fat and fibrous tissue that may result from chronic myocarditis and also distinct from the strands of cardiomyocytes found in fatty tissue without fibrosis, which could be a normal variant10; sparing of subendocardial myocardium which may show trabecular hypertrophy or disarrangement (Fig. 1); substantial sparing of the left ventricular myocardium; and the absence of other cardiac diseases. These criteria were present in all the patients studied. Extensive mononuclear infiltrates and diffuse interstitial fibrosis superimposed on the typical pattern of arrhythmogenic right ventricular dysplasia were observed on histologic analysis in only

Sections from the right ventricle of four normal subjects (three men and one woman; mean age, 41 ± 16 years) who died of other, noncardiac causes served as controls. None of these control subjects met any of the histologic criteria for right ventricular dysplasia. Although the interval between death and autopsy did not affect the detection of apoptosis, only cases in which this interval was 24 hours or less were included. Tissues were fixed in 10 percent buffered formalin and embedded in paraffin. Four to six sections (6 μ m thick) from each paraffin block were analyzed for the presence of apoptosis.¹³

In Situ Detection of Apoptotic Cells

Sections were deparaffinized, transferred to xylene, and rehydrated in descending concentrations of alcohol (100 percent,

From the Centre de Rythmologie et de Stimulation Cardiaque, Hôpital Jean Rostand, Ivry-sur-Seine (Z.M., F.F., R.F., G.F.); INSERM Unité 141, Hôpital Lariboisière, Paris (Z.M., A.T.); and the Service d'Anatomie et de Cytologie Pathologiques, Hôpital Raymond Poincaré, Garches (M.D.) — all in France. Address reprint requests to Dr. Mallat at the Centre de Rythmologie et de Stimulation Cardiaque, Hôpital Jean Rostand, 39, rue Jeanle-Galleu, 94200 Ivry-sur-Seine, France.

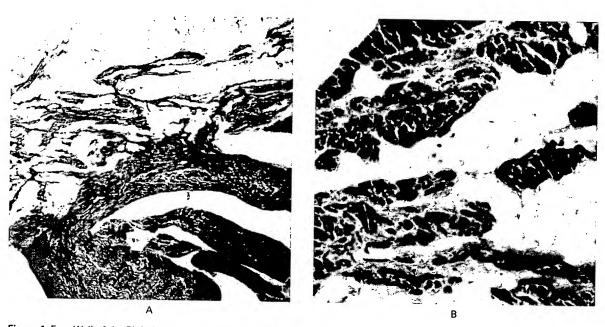


Figure 1. Free Wall of the Right Ventricle of a Patient with Right Ventricular Dysplasia.

In Panel A, there is a large amount of adipose tissue occupying the mediomural and subepicardial layers (hematoxylin-phloxin-safran staining, ×10). In Panel B, high magnification reveals surviving strands of myocardium bordered by or embedded in fibrous tissue. The presence of fibrous tissue is necessary for the diagnosis of right ventricular dysplasia¹⁰ (hematoxylin-phloxin-safran staining, ×100).

95 percent, 70 percent, 50 percent, and 0 percent). After rehydration, the slides were incubated with 20 μ g of proteinase K per milliliter in phosphate-buffered saline. Endogenous peroxidase was inactivated by 3 percent hydrogen peroxide. Tissue sections were stained with an ApopDETEK system (Enzo Diagnostics, Farmingdale, N.Y.) that identifies cells with internucleosomal fragmentation of DNA (apoptosis). The procedure was performed according to the manufacturer's instructions. The method is based on the preferential binding of terminal deoxynucleotidyl transferase (TdT) to the 3'-hydroxyl ends of DNA.13 Briefly, residues of biotinylated deoxyuridine triphosphate (dUTP) were catalytically added to the ends of DNA fragments with the enzyme TdT. For negative controls, deionized water was used instead of TdT. After end-labeling, the sections were incubated with avidin-horseradish peroxidase and stained with diaminobenzidine to detect the biotin-labeled nuclei. Apoptotic bodies stained brown. Positive controls consisted of rat mammary glands obtained on the fourth day after weaning (Oncor, Gaithersburg, Md.). Four to six sections from each specimen were examined. Sections were first examined under light microscopy at low magnification (×100), allowing estimation of the percentage of surface area occupied by apoptotic cells. Then, 10 random fields per section from the regions with apoptotic cells were examined at a higher magnification (×400) to calculate the percentage of myocardial nuclei with DNA fragmentation. Cardiomyocytes, which were well-shaped, elongated, and striated cells, were easily distinguished morphologically from other rare nonmyocytes under a light microscope at high magnification. In addition to the in situ end-labeling technique, adjacent sections stained with hematoxylin and eosin were examined for signs of apoptosis.14 The pathologist analyzing the specimens was unaware of the diagnosis in 9 of the 12 cases examined.

Immunohistochemical Detection of Protease CPP-32

CPP-32 is a cysteine protease required for the initiation of apoptotic cell death. If it is related to interleukin- 1β -converting en-

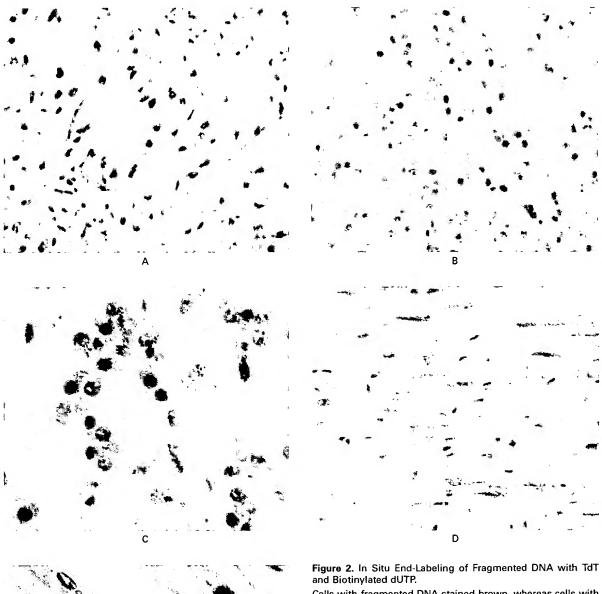
zyme (ICE) and CED-3, the product of a gene required for programmed cell death in the nematode *Caenorhabditis elegans*. CPP-32 is the specific ICE/CED-3-like mammalian cysteine protease that cleaves and inactivates poly(adenosine diphosphate ribose) polymerase, an enzyme involved in DNA repair and genome integrity, and thus may be the human equivalent of CED-3.15 Therefore, to provide further evidence of the occurrence of apoptosis in arrhythmogenic right ventricular dysplasia, we analyzed the level of expression of CPP-32 in the right ventricles of the patients and controls using immunohistochemical techniques.

After deparaffinization and rehydration, the sections were incubated with 1:10 normal horse serum for 30 minutes at room temperature, washed once in phosphate-buffered saline, and stained with a mouse monoclonal anti-CPP-32 antibody (Transduction Laboratories, Lexington, Ky.) at a dilution of 1:1000. The slides were washed in phosphate-buffered saline and then incubated with biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, Calif.) at a dilution of 1:200. Stains were visualized with an avidin-alkaline phosphatase-substrate system (Vectastain ABC Kit and Vector Red, Vector Laboratories, Burlingame, Calif.). As a negative control, serial sections were stained without the primary antibody against CPP-32.

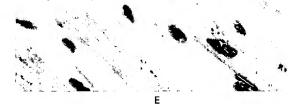
RESULTS

Evidence of Apoptosis

In situ end-labeling of fragmented DNA with TdT and biotinylated dUTP did not reveal apoptosis in sections of right ventricular myocardium from the four normal adult subjects (Fig. 2A). In contrast, sections of right ventricular myocardium from six of the eight patients with arrhythmogenic right ventricular dysplasia showed numerous cells with genomic DNA fragments in their nuclei (Fig. 2B, 2C,



Cells with fragmented DNA stained brown, whereas cells with normal nuclei stained blue (immunoperoxidase staining with hematoxylin counterstaining). In Panel A, a section from a normal human right ventricle shows no apoptotic nuclei (×100). Transverse sections (Panels B and C) and longitudinal sections (Panels D and E) of right ventricular myocardium from patients with lethal arrhythmogenic right ventricular dysplasia show numerous myocardial nuclei with apoptosis. (Panels B and D, ×100; Panels C and E, ×400.)



2D, and 2E). The majority of these cells were easily recognized as myocardial cells under a light microscope at high magnification, since they were well shaped, elongated, and striated (Fig. 2C and 2E). The apoptotic myocardial cells were frequently in regions of myocardium not already invaded by adipocytes and fibrosis. They were less frequently in regions replaced by fat and fibrous tissue, where rare, nonapoptotic cardiomyocytes were still present. Both the extent of regions with apoptotic cells and the percentage of apoptotic myocardial cells in these regions varied among the patients (Table 1). An inflammatory reaction was detected in sections from one of the eight patients (Patient 1). This patient had the highest percentage of apoptotic myocardial cells, and some inflammatory cells were also apoptotic (Table 1 and Fig. 3).

The detection of apoptotic cells by in situ endlabeling of fragmented DNA was supported by the fact that pathological criteria for apoptosis were also met. In adjacent sections stained with hematoxylin and eosin, nuclei of numerous myocardial cells showed marginated masses of chromatin along with discrete, well-preserved apoptotic bodies (Fig. 4), typical pathological features of apoptosis. ¹⁴ No sign of apoptosis was seen in adjacent sections stained with hematoxylin and eosin from the four normal subjects (data not shown).

Expression of CPP-32 in Right Ventricular Myocardium from Patients with Arrhythmogenic Right Ventricular Dysplasia

Protease CPP-32 is important for the induction of apoptotic cell death in mammalian cells.¹⁵ It was undetectable or barely detectable in the right ventricles of the four normal subjects (Fig. 5A), as well as in the two patients with no evidence of apoptosis. However, cardiomyocytes from the right ventricles of the six patients with apoptosis showed high levels of immunoreactive CPP-32 (Fig. 5B). No staining was detected after omission of the primary anti–CPP-32 antibody.

DISCUSSION

We report the occurrence of apoptotic myocardial cell death in right ventricular dysplasia. James⁶ has previously suggested that apoptosis may be a mechanism of cell death in arrhythmogenic right ventricular dysplasia.

In the present study, apoptosis was identified by in situ end-labeling of fragmented DNA with TdT and biotinylated dUTP, a commonly accepted method for the detection of the apoptotic process. There has been concern about the ability of such in situ labeling methods to distinguish between cell necrosis and apoptosis. However, in this study the detection of a positive reaction with in situ end-labeling was correlated with the presence of typical

TABLE 1. EXTENT OF AREAS OF APOPTOSIS AND THE PERCENTAGE OF APOPTOTIC MYOCARDIAL NUCLEI IN THESE AREAS IN SECTIONS OF RIGHT VENTRICULAR MYOCARDIUM FROM PATIENTS WITH LETHAL ARRHYTHMOGENIC RIGHT VENTRICULAR DYSPLASIA.*

Patient No.	Extent of Areas with Apoptotic Nuclei	PROPORTION OF APOPTOTIC NUCLEI IN POSITIVE AREAS
	% of section	%
1	50	28
2	20	15
3	10	18
4	20	20
5	0	0†
6	15	22
7	15	14
8	0	0†

*Four to six sections from each specimen were examined. After in situ end-labeling of fragmented DNA, sections were examined under light microscopy at low magnification (×100), allowing an estimation of the surface area occupied by apoptotic cells. Then, 10 random fields per section from these positive areas were examined at high magnification (×400) to calculate the percentage of myocardial nuclei with DNA fragmentation.

†Only one apoptotic cell per section was found.

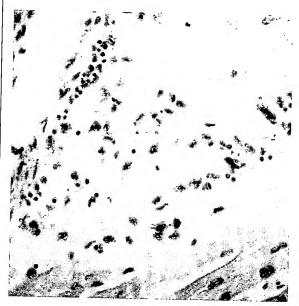


Figure 3. Apoptotic Nuclei in Nonmyocytes in an Inflammatory Reaction in One Patient (Immunoperoxidase Staining with Hematoxylin Counterstaining, ×250).

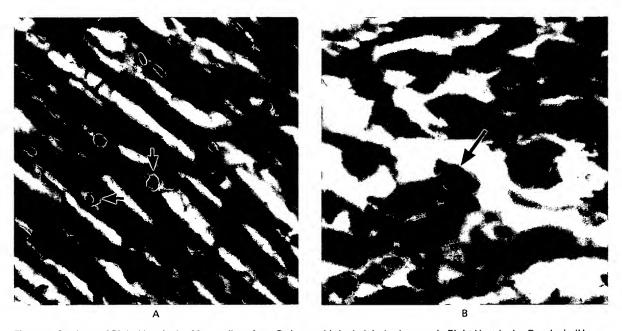


Figure 4. Sections of Right Ventricular Myocardium from Patients with Lethal Arrhythmogenic Right Ventricular Dysplasia (Hematoxylin and Eosin).

In Panel A, there are marginated masses of chromatin within myocardial nuclei in a longitudinal section (arrows) (×250). In Panel B there are multiple round, hyperdense nuclear fragments, whose appearance is consistent with that of apoptotic bodies, in a transverse section (arrow) (×400).

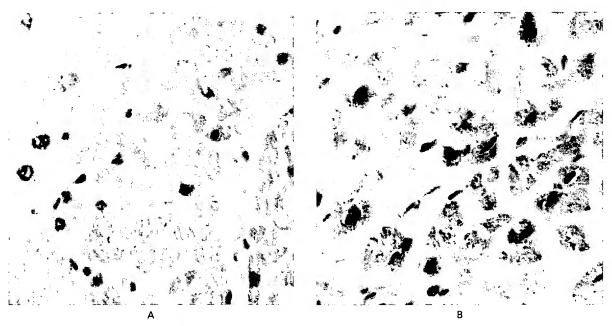


Figure 5. Immunohistochemical Detection of CPP-32.

In Panel A, normal right ventricular myocardium does not stain for CPP-32 (×400). In Panel B, right ventricular myocardium from a patient with right ventricular dysplasia stains intensely for CPP-32 (×400). The antibody against CPP-32 was detected with antimouse IgG conjugated with biotin and an avidin-alkaline phosphatase-substrate system in which positive cells stain red.

signs of apoptosis (marginated masses of chromatin and well-preserved apoptotic bodies) in sections stained with hematoxylin and eosin. Moreover, the absence of an inflammatory reaction in most of our patients argues against necrosis and for apoptosis. Furthermore, no positive staining was detected in cardiomyocytes from age-matched normal subjects whose hearts were processed in the same manner as those of our patients, and the in situ end-labeling of fragmented DNA was not detected when the enzyme TdT was omitted.

To gain further evidence of apoptosis in arrhythmogenic right ventricular dysplasia and extend our findings, we examined the level of expression of protease CPP-32, whose activation is specifically required for apoptotic cell death in mammalian cells. Our finding that high levels of CPP-32 expression were associated with positive in situ end-labeling of fragmented DNA provides strong evidence of apoptotic cell death in arrhythmogenic right ventricular dysplasia.

We found that numerous cells in the right ventricle of patients with arrhythmogenic right ventricular dysplasia underwent apoptosis. The majority of these cells were morphologically identified as myocardial cells under high-power magnification. The affected areas had few or no apoptotic cells, whereas apoptotic nuclei were frequently seen in areas with little involvement. This finding suggests that the loss of myocardial cells through apoptosis is, at least in part, a primary process that precedes the filling of acellular space by fat and fibrous tissue in the absence of an inflammatory reaction.

The extent and the percentage of apoptotic myocardial cells varied among the patients. The absence of apoptosis (only one apoptotic cell per section) in two of our patients is intriguing. These patients' clinical and histologic features were similar to those of the other patients. Apoptosis may therefore not have been involved in the pathogenesis of arrhythmogenic right ventricular dysplasia in these patients. However, the possibility that most of the apoptotic cells had already been cleared by the time the heart sections were obtained cannot be ruled out. Studies of the clearance kinetics of apoptotic myocardial cells should clarify this issue.

The triggering factors for apoptotic myocardial cell death in arrhythmogenic right ventricular dysplasia remain to be elucidated. Some evidence from in vitro and in vivo studies in animals suggests that hypoxia as well as reperfusion injury are possible triggers for apoptosis in cardiomyocytes.^{17,18} These factors may also contribute to the induction of apoptosis in myocardial cells of the failing canine heart.¹⁹ Repeated ventricular arrhythmias may have produced an ischemia–reperfusion injury and contributed to the apoptotic process in our patients. The presence of myocarditis (and its related production of inflammatory cytokines) could also have had a role in the

induction or aggravation of the apoptotic process. However, myocarditis is not a consistent or prominent feature of arrhythmogenic right ventricular dysplasia, and only one of our patients had associated myocarditis. Abnormal levels of resting tension, which could result from the architectural rearrangement of the myocyte compartment in the diseased right ventricle, could also have contributed to the activation of the suicide program in these cells. ²⁰ Finally, primary abnormal control of genes involved in the regulation of programmed cell death — for instance, *CPP-32* — remains plausible.

In conclusion, we found that numerous myocardial cells from the right ventricles of a majority of patients with lethal arrhythmogenic right ventricular dysplasia actively undergo programmed cell death. This finding could account, at least in part, for the progressive loss of myocardial cells observed in this disease and may shed new light on its pathogenesis.

Supported by the Association pour l'Etude des Cardiomyopathies and by an award from Assistance Publique, Hôpitaux de Paris (to Dr. Mallat).

We are indebted to Dr. Jane-Lyse Samuel for helpful discussion of the manuscript.

REFERENCES

- 1. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26:239-57.
- 2. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980;68:251-306.
- 3. Walker NI, Harmon BV, Gobé GC, Kerr JFR. Patterns of cell death. Methods Achiev Exp Pathol 1988;13:18-54.
- 4. Williams GT, Smith CA. Molecular regulation of apoptosis: genetic controls on cell death. Cell 1993;74:777-9.
- Carson DA, Ribeiro JM. Apoptosis and disease. Lancet 1993;341:1251-
- **6.** James TN. Normal and abnormal consequences of apoptosis in the human heart: from postnatal morphogenesis to paroxysmal arrhythmias. Circulation 1994;90:556-73.
- James TN, Nichols MM, Sapire DW, DiPatre PL, Lopez SM. Complete heart block and fatal right ventricular failure in an infant. Circulation 1996; 93:1588-600.
- 8. Marcus FI, Fontaine GH, Guiraudon G, et al. Right ventricular dysplasia: a report of 24 adult cases. Circulation 1982;65:384-98.
- 9. Thiene G, Nava A, Corrado D, Rossi L, Pennelli N. Right ventricular cardiomyopathy and sudden death in young people. N Engl J Med 1988; 318:129-33.
- 10. Fontaliran F, Fontaine G, Fillette F, Aouate P, Chomette G, Grosgogeat Y. Frontières nosologiques de la dysplasie arythmogène: variations quantitatives du tissu adipeux ventriculaire droit normal. Arch Mal Coeur 1991; 84:33-8.
- 11. McKenna WJ, Thiene G, Nava A, et al. Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy: Task Force of the Working Group Myocardial and Pericardial Disease of the European Society of Cardiology and of the Scientific Council on Cardiomyopathies of the International Society and Federation of Cardiology. Br Heart J 1994;71: 215-8.
- 12. Fontaine G, Fontaliran F, Andrade FR, et al. The arrhythmogenic right ventricle: dysplasia versus cardiomyopathy. Heart Vessels 1995;10: 227-35.
- 13. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493-501.
- 14. Kerr JFR, Winterford CM, Harmon BV. Morphological criteria for identifying apoptosis. In: Celis JE, ed. Cell biology: a laboratory handbook. Vol. 1. San Diego: Academic Press, 1994:319-29.
- 15. Nicholson DW, Ali A, Thornberry NA, et al. Identification and inhi-

bition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995;376:37-43.

- **16.** Gold R, Schmied M, Giegerich G, et al. Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. Lab Invest 1994;71:219-25.
- 17. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 1994:94:1621-8.
- **18.** Gottlieb RA, Gruol DL, Zhu JY, Engler RL. Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis. J Clin Invest 1996;97:2391-8.
- 19. Sharov VG, Sabbah HN, Shimoyama H, Goussev AV, Lesch M, Goldstein S. Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure. Am J Pathol 1996;148:141-9.
- 20. Cheng W, Li B, Kajstura J, et al. Stretch-induced programmed myocyte cell death. J Clin Invest 1995;96:2247-59.

IMAGES IN CLINICAL MEDICINE

Images in Clinical Medicine, a weekly Journal feature, presents clinically important visual images, emphasizing those a doctor might encounter in an average day at the office, the emergency department, or the hospital. If you have an original unpublished, high-quality color or black-and-white photograph representing such a typical image that you would like considered for publication, send it with a descriptive legend to Karen Pedersen, New England Journal of Medicine, 10 Shattuck St., Boston, MA 02115. For details about the size and labeling of the photographs, the requirements for the legend, and authorship, please contact Karen Pedersen at 617-734-9800 (phone) or 617-739-9864 (fax), or the New England Journal of Medicine at images@edit.nejm.org (e-mail).

THIC PAGE BI ANK (USPTO)